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MicroRNAs (miRNAs) are a class of single-stranded molecules of 18-22 nt length with increasing evidence of having a crucial regulatory role in posttranscriptional gene expression regulation. These single-stranded miRNAs bind to complementary sites mostly on the 3' untranslated region (UTR) of target genes, and consequently regulate post-transcriptional gene expression by mRNA degradation and translational repression. They act in a very dynamic and subtle way in regulation, one miRNA can regulate hundreds of different targets; and more than one miRNA may coordinately regulate a single target. Their role is well known in several key biological processes, maintaining organismal homeostasis and developmental timing. A still emerging area is to identify them as key factors involved in cancer at all stages ranging from initiation to metastasis, their role in other pathologies and using them as a diagnostic tool and treatments for a disease.

To address the challenge of studying microRNA-based regulatory processes we chose the *in vivo* approach using the popular model organism *Drosophila melanogaster*. The fruit fly represents an ideal animal model to study microRNAs, as gene redundancy is much lower than in mammals, simplifying functional analyses. There are more than 1500 microRNAs in human. The 240 such genes found in fruit flies include all major microRNA families, and clear homology of a substantial subset of Drosophila and human microRNAs is evident.

The initial research question was to identify regulator molecules of autophagy, carrying out a large-scale *in vivo* screen in Drosophila. In the screen a new collection of microRNA mutants were used that were made by targeted knockout mutations, providing a great resource for large-scale analysis. This collection of 80 new Drosophila miRNA mutants remove 104 miRNAs, which account for the 99% of Drosophila miRNA sequence reads, and more than 80% of the mutants produced an observable phenotype in the published tests.

The screen was based on widely used biochemical autophagy tests, on the detection of Atg8a present on autophagosomes, and p62 to measure the levels of the most common selective autophagic cargo. I used our self-made antibodies on immunoblots. Starvation-induced reduction in p62 levels is not observed in autophagy-deficient cells, and instead, p62 accumulates. The screen provided information about autophagy defects observed in the examined mutants.

One of the most convincing hits was dmel-miR-995, which is the still uncharacterized member of the human miR-29 oncomiR family, based on seed sequence homology. MiR-995 knockout mutants have a shortened lifespan, neuromuscular defects and the cells lack starvation-induced autophagic structures in the fat body (the analog of human liver and fat tissues specialized for energy storage and metabolism), which suggests that the initiation of the whole process is blocked. Moreover, the lack of miR-995 results in an increased body size, while cell-autonomous overexpression leads to small cells and negatively affects organismal growth. The central regulator of growth and metabolism in eukaryotic cells is TOR kinase that integrates information from several sources about the nutritional status of the organism. As an important output, this protein regulates protein synthesis acting on the translational regulators 4E-BP and S6K proteins and induces autophagy upon the lack of nutrients. Further genetic tests underlined that miR-995 might be involved *in vivo* in repressing TOR activity. Despite previous extensive database search for seed sequence homology among potential target proteins, we could not detect changes nor in the mRNA nor in the protein levels of the numerous potentially involved proteins that we tested.

The change in the regulation of nutritional status sensing was also apparent in other tissues in miR-995 loss and gain-of-function animals. Moreover, we identified striking differences in the morphology of the dissected GI (gastro-intestinal) tract of miR-995 mutants. In addition, we observed that miR-995 mutants do not eliminate ingested pathogenic bacteria like healthy controls highlighting that this miRNA might impact intestinal immunity and defensive pathways. Intestinal food passage was also decreased in miR995 mutants meaning that gut retention of food, bacteria etc. increased in the absence of this miRNA. These phenotypes were completely reverted in the presence of a miR995 rescue construct confirming its function in gut physiology. More specifically, we

identified that the level of miR-995 in the gut fine tunes Notch signaling activity in intestinal stem cells (ISC) to balance stem cell decision and proliferation. Interestingly, miR995 seems to coregulate neuralized and snail in ISCs, two essential factors for maintaining stem cell behavior. We verified this after extensive cloning and popular transgenic fly generation steps by utilizing in vivo GFP sensors to measure miRNA expression in ISCs. We believe that this protocol is the most reliable way to demonstrate miRNA functionality in vivo in a tissue and in a specific cell type. We extended our analyses to miRNA-285 because elevated level of human miRNAs mapped into the miR-29 superfamily were recently identified as a biomarker for colorectal cancer (CRC). miR-285 overexpression and deletion were a clear phenocopy of miR-995. Furthermore, we demonstrated that stem cell-specific overexpression of miR-995 activated general stress- (JNK and Upd3/JAK-STAT) and developmental (Vein/EGF) signaling pathways in the gut. Thus, we believe that our results about miR-995/285 will help to understand the function and mechanism of miR-29 superfamily in humans to pave the way for developing new strategies of CRC therapies, prevention and diagnoses.

With the incorporation of collaboration partners we can test our data and the role of this microRNA family in the recently developed new 3-D culture system known as organoid culture that resembles the properties of the original tissues. Considering the fact that colorectal cancer is among the highest cause of cancer occurrence and cancer-related death worldwide, our findings are hopefully of great potential interest. The data is ready for publication and we plan to submit the manuscript this year upon having collaborators' data.

During these years I have also continued my previously established project on the investigation of the cellular and molecular aspects of autophagy research that resulted in a first author publication in 2016 (Hegedus et al., Mol Biol Cell 27:(20) pp. 3132-3142.). Furthermore, I participated in various projects in the field, which resulted in several co-authored publications in high impact peer-reviewed journals (see publication list at the end).

Membrane trafficking events are regulated by Rab family small GTPases. Among critical regulators of vesicular trafficking, Rab5 and Rab7 play central roles in the maturation of early to late endosomes and endolysosomes. Although numerous Rab proteins have

been implicated in autophagy, it is still unclear which of them play central roles in this process.

In our study we showed that the Rab7 module and Rab5 control different steps of autophagy. Rab7 mediates autophagosome-lysosome fusion together with its GEF, the Ccz1-Mon1 complex. This is likely achieved by the recruitment of Rab7 to autophagosomes in a Ccz1-Mon1-dependent manner. Although *Drosophila* Mon1 binds to the active, GTP-locked form of Rab5 as in other organisms, Rab5 is dispensable for the fusion of autophagosomes with lysosomes, and for Rab7 localization to autophagosomes and autolysosomes. The question was then: what is the signal that recruits Ccz1-Mon1 and Rab7 to autophagic structures? Mon1 and Ccz1 bind to phospholipids including PI3P in yeast, and we found that Drosophila Mon1 has similar features. This raises the possibility that the Ccz1-Mon1 complex is recruited to the PI3P-positive surface of autophagosomes through this interaction. Vps34-dependent PI3P generation is required for autophagosome formation and endosome maturation. Vps34 is activated by Rab5. Interestingly, our data suggested that loss of Rab5 inhibits PI3P generation only on endosomes, but not on autophagosomes. Loss of UVRAG but not Atg14 inhibits PI3P generation on endosomes, while loss of Atg14 leads to complete inhibition of PI3P positive autophagosome biogenesis. Thus, UVRAG is dispensable for Vps34 activity during autophagosome formation, and its loss causes a defect in autolysosomal degradation. Similarly, Rab5 mutant cells showed accumulation of autophagic cargo due to impaired lysosomal degradation.

Based on our results, we proposed the following model of autolysosome formation in fat cells of starved Drosophila larvae: PI3P-positive autophagosomes are generated through the action of an Atg14-containing Vps34 PI3 kinase complex. PI3P attracts Ccz1-Mon1, which promotes Rab7 recruitment to autophagosomes. Both PI3P and Rab7 bind to the HOPS tethering complex, and thus these factors promote the tethering of autophagosomes with late endosomes and lysosomes. The membrane fusion is then executed by the Syx17-Snap29-Vamp7 SNARE complex. Autophagic cargo is broken down in autolysosomes, and their full degradative capacity requires the function of Rab5 and the UVRAG-containing Vps34 complex for the proper delivery of lysosomal proteins, likely including both acidic hydrolases and membrane proteins.

We proposed that autophagosomal PI3P recruits the Ccz1-Mon1-Rab7module to facilitate the loading of HOPS and subsequent tethering of vesicles. We found that while Rab5 only mediates the generation of PI3P on endosomes mainly through the action of a UVRAG-containing Vps34 complex, it is dispensable for PI3P-positive autophagosome biogenesis that depends on the Atg14-containing Vps34 complex. So the current concept that Vps34 is a Rab5 effector must be revisited: it is true for endocytosis but not applicable for autophagy in fat cells of starved Drosophila larvae.

List of publications during funding period (2014-2017)

Peer-reviewed scientific publications (journals)

1. Nagy P, Szatmari Zs, Sandor Gy O, Lippai M, Hegedus K and Juhász G
   Drosophila Atg16 promotes enteroendocrine cell differentiation via regulation of intestinal Slit/Robo signaling
   DEVELOPMENT 2017 doi:10.1242/dev.147033 (accepted for publication)
   IF:5.843
2. Csizmadia T, Lorincz P, Hegedus K, Szeplaki Sz, Low P and Juhasz G
   Molecular mechanisms of developmentally programmed crinophagy in Drosophila
   JOURNAL OF CELL BIOLOGY 2017 (accepted for publication)
   IF: 9.688
3. Lorincz P, Toth S, Benko P, Lakatos Z, Boda A, Glatz G, Zobel M, Bisi S, Hegedus K, Takats S, Scita G, and Juhasz G
   Rab2 promotes autophagic and endocytic lysosomal degradation.
   JOURNAL OF CELL BIOLOGY 2017 May 8. pii: jcb.201611027 DOI: 10.1083/jcb.201611027
   IF: 9.688
4. Hegedus K, Takats S, Boda A, Jipa A, Nagy P, Varga K, Kovacs AL, Juhasz G
   The Ccz1-Mon1-Rab7 module and Rab5 control distinct steps of autophagy.
   MOLECULAR BIOLOGY OF THE CELL 27:(20) pp. 3132-3142. (2016)
   IF:4.548
5. Lorincz P, Lakatos Z, Varga A, Maruzs T, Simon-Vecsei Z, Darula Z, Benko P, Csordas G, Lippai M, Ando I, Hegedus K, Medzihradszky K, Takats S, Juhasz G

MiniCORVET is a Vps8-containing hemocyte- and nephrocyte-specific early endosomal tether in Drosophila

ELIFE 5: Paper e14226. 27 p. (2016)

IF: 8.734

6. Papp D, Kovacs T, Billes V, Varga M, Tarnoci A, Hackler L Jr, Puskas LG, Liliom H, Tarnok K, Schlett K, Borsy A, Padar Z, Kovacs AL, Hegedus K, Juhasz G, Komlos M, Erdos A, Gulyas B, Vellai T

   AUTEN-67, an autophagy-enhancing drug candidate with potent antiaging and neuroprotective effects.

   AUTOPHAGY 12:(2) pp. 273-286. (2016)

   IF: 11.753

7. Varga K, Nagy P, Arsikin Csordás K, Kovács AL, Hegedűs K, Juhász G

   Loss of Atg16 delays the alcohol-induced sedation response via regulation of Corazonin neuropeptide production in Drosophila

   SCIENTIFIC REPORTS 6: Paper 34641. 10 p. (2016)

   IF: 5.578

8. Hegedus K, Nagy P , Gaspari Z , Juhasz G

   The Putative HORMA Domain Protein Atg101 Dimerizes and Is Required for Starvation-Induced and Selective Autophagy in Drosophila

   BIOMED RESEARCH INTERNATIONAL 2014: Paper 470482. 13 p.(2014)

   IF: 2.706

9. Nagy P , Karpati M , Varga A , Pircs K , Venkei Z , Takats S , Varga K , Erdi B , Hegedus K , Juhasz G

   Atg17/FIP200 localizes to perilysosomal Ref(2)P aggregates and promotes autophagy by activation of Atg1 in Drosophila

   AUTOPHAGY 10:(3) pp. 453-467. (2014)

   IF: 11.753

10. Nagy P , Hegedus K , Pircs K , Varga A , Juhasz G

    Different effects of Atg2 and Atg18 mutations on Atg8a and Atg9 trafficking during starvation in Drosophila.

    FEBS LETTERS 588: pp. 408-413. (2014)

    IF: 3.169

11. Szatmari Z , Kis V , Lippai M , Hegedus K , Farago T , Lorincz P , Tanaka T , Juhasz G , Sass M
    Rab11 facilitates crosstalk between autophagy and endosomal pathway through regulation of Hook localization.
    MOLECULAR BIOLOGY OF THE CELL 25:(4) pp. 522-531. (2014)
    IF: 4.548
12. Takats S , Pircs K , Nagy P , Varga A , Karpati M , Hegedus K , Kramer H , Kovacs AL , Sass M , Juhasz G
    Interaction of the HOPS complex with Syntaxin 17 mediates autophagosome clearance in Drosophila
    MOLECULAR BIOLOGY OF THE CELL 25:(8) pp. 1338-1354. (2014)
    IF: 4.548